Generation of Genome-Edited Chicken Through Targeting of Primordial Germ Cells

Generation of genome-edited chicken through targeting of primordial germ cells

Alewo Idoko-Akoh[1] and Michael J McGrew[1]

[1]The Roslin Institute & Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Campus, Midlothian EH25 9RG, United Kingdom.

Corresponding author: Michael J. McGrew mike.mcgrew@roslin.ed.ac.uk

Abstract

Genome editing technology facilitates the creation of specific and precise genetic changes to unravel gene function and rapidly transfer unique alleles between chicken breeds in contrast to lengthy traditional crossbreeding methods for the study of poultry genetics. Innovations in genome sequencing technology have made it possible to map polymorphisms associated with both monogenic and multigenic traits in livestock species. We, and many others, have demonstrated the use of genome editing to introduce specific monogenic traits in chicken through targeting of cultured primordial germ cells. In this chapter, we describe materials and protocols for performing heritable genome editing in the chicken through targeting of in vitro propagated chicken primordial germ cells.

1 Introduction

In 2015, the Avian Phyologenetics Consortium initiated a project to sequence the genomes of 10,000 bird species (Zhang, 2015). Since then, ongoing technological developments in genome sequencing has led to the generation of over 500 avian reference genomes as at the time of writing this manuscript (Bravo et al., 2021). This development in avian genomics presents the opportunity to exploit the application of genome editing technology to investigate gene regulatory function, perform rapid allele transfer between breeds and introduce novel alleles into bird species. The methods for performing precise genome editing in zygotes are well described for many mammalian species but are not readily applicable in birds due to evolutionary differences in reproductive biology. The development of methods for *in vitro* propagation of chicken primordial germ cells (PGCs) was a significant step in actualizing defined and heritable genetic modification in avian species (van de Lavoir et al., 2006)**.** PGCs present a cell lineage that can be targeted to introduce genetic changes that will be heritable in offspring derived from the modified germ cells. We and others have demonstrated the generation of genome-edited chickens from targeted cultured chicken PGCs (Oishi et al., 2016; Park et al., 2014; Taylor et al., 2017). CRISPR/Cas9-aided gene editing has been used to perform seamless a footprint-less transfer of monogenic traits between chicken breeds (Ballantyne et al., 2021), and to study genes that control avian germ cell and gonadal development (Ioannidis et al., 2021; Lee et al., 2017). We previously described a serum-free and feeder-free optimized medium suitable for chicken PGC derivation, long-term PGC culture and single cell culture for clonal derivations (Whyte et al., 2015). The use of this optimized medium eliminates the risk of pathogen contamination associated with animal-derived products. We have also generated sterile i-Caspase9 sterile surrogate hosts through genome editing of PGCs to facilitate the generation of offspring with homozygous for the desired genotype genetic change in a single breeding generation (Ballantyne et al., 2021).

In this chapter, we will describe methods for the derivation and genome editing of chicken PGCs in serum-free, feeder-free medium to generate clonal targeted PGCs and subsequent PGC injection into surrogate host embryos. The methods described in this chapter may be applied in other avian species if culture methods for the PGCs from these species are developed. However, the application of genome editing using cultured PGCs for the majority of other avian species is still not possible. The overall pathway to proceed from embryo to cultured PGC, to genome edited PGC, to surrogate host chicken carrying the engineered chicken germ cells is shown in **Figure 1**.

2 Materials

2.1 Avian KnockOut DMEM (KO-DMEM): a no-calcium, low osmolarity DMEM for culturing embryonic cells.

1. Avian KO-DMEM (Life Technologies 041-96570M) or
2. Alternatively, prepare ~50 ml of avian KO-DMEM replacement as follows:

a. 37.5 ml of calcium-free DMEM (Life Technologies: 21068028).
b. 11.6 ml of distilled water (Life Technologies: 15230089).
c. 0.5 ml of 50X MEM amino acids solution (Life Technologies: 11130051).
d. 0.5 ml of 100 mM Sodium Pyruvate (Life Technologies: 11360070).
e. 0.5 ml of 100X MEM vitamin solution (Life technologies: 11120052).

2.2 PGC basal medium

Prepare 50 ml of PGC basal medium as follows:

1. 47 ml of avian KO-DMEM.
2. 1 ml of 50X B27 supplement (Life Technologies: 17504044).
3. 0.5 ml of 100X MEM non-essential amino acids (Life Technologies: 11140050).
4. 0.5 ml of 100X GlutaMAX™-I (Life Technologies: 35050061).
5. 0.5 ml of 100X EmbryoMax® nucleosides (Merck Millipore: ES-008-D). (**See Note 1**)
6. 0.2 ml of 100 mM Sodium Pyruvate (Life Technologies: 11360070).
7. 0.1 ml of 50 mM 2-Mercaptoethanol (Life Technologies: 31350010).
8. 0.075 ml of 100 mM $CaCl_2$ dissolved in tissue-culture grade distilled water. Use solution filtered through a 0.22 µm syringe filter (Merck Millipore; SLGPO33RS).
9. 0.5 ml of 20% Ovalbumin (Sigma-Aldrich: A5503). Prepare 5 ml of the solution as follows:
    a. Add 1000 mg of ovalbumin to 5 ml of KO-DMEM.
    b. Put on a roller for up to 20 minutes or until the solution is clear for complete dissolution.
    c. Filter the clear solution through a 0.22 µm syringe filter.
    d. Aliquot and store at 4˚C.
10. 0.1 ml of 50 mg/ml Heparin sodium (Sigma-Aldrich: H3149). Prepare 5 ml of the solution as follows:
    a. Add 250 mg of heparin sodium to 5 ml of KO-DMEM.
    b. Put on a roller for up to 20 minutes or until the solution is clear for complete dissolution.
    c. Filter the clear solution through a 0.22 µm syringe filter.
    d. Aliquot and store at 4˚C.
11. 0.1 ml of penicillin/streptomycin (10,000 U/ml) (Life Technologies: 15140122). (optional)

2.3 5000X Vitamin B12 (Sigma-Aldrich: V6629) prepared as follows (see Note 2):

1. Prepare 500,000X stock (3.4 mg/ml): dissolve 100 mg of Vitamin B12 in 29.41 ml tissue-culture grade distilled water.
2. Filter the stock solution through a 0.22 µm syringe filter and store at -20˚C.
3. Prepare 5000X stock (0.034 mg/ml): add 100 µl of the 500,000X stock solution to 10 ml of tissue-culture grade distilled water.
4. Filter the solution through a 0.22 µm syringe filter and store at -20˚C.

2.4 PGC growth factors

1. Recombinant human FGF2 (R & D systems: 234-FSE-025) is shipped on dry ice as a lyophilised powder containing Tris-NaCl and bovine serum albumin (BSA) as a carrier. Prepare in a sterile tissue culture hood as follows:
    a. Spin 25-µg FGF2 vial for 15 seconds in a centrifuge.
    b. Filter tissue-culture grade phosphate buffered saline (PBS) through a 0.22 µm syringe filter into a sterile 15 ml conical tube.
    c. Add 5 µl of sterile 20% ovalbumin to 1 ml of sterile PBS to prepare a 0.1% ovalbumin/PBS solution.
    d. Add 1 ml of 0.1% ovalbumin/PBS solution to the 25-µg FGF2 vial to achieve a stock concentration of 25 µg/ml. (**see Note 3**)
    e. Allow to stand for 10 minutes, and then pipette up and down six times.
    f. Make aliquots and store at -80˚C. Upon thawing, do not refreeze and use within 4 weeks.
2. Recombinant human Activin A (Peprotech: 120-14). Prepare in a sterile tissue culture hoods as follows:
    a. Spin 5-µg Activin A vial for 15 seconds in a centrifuge.
    b. Filter tissue-culture grade PBS through a 0.22 µm syringe filter into a sterile 15 ml conical tube.

c. Add 50 µl of sterile 20% ovalbumin to 10 ml of sterile PBS to prepare a 0.1% ovalbumin/PBS solution.
d. Add 200 µl of 0.1% ovalbumin/PBS solution to the 25-µg FGF2 vial to achieve a stock concentration of 25 µg/ml.
e. Allow to stand for 10 minutes, and then then pipette up and down six times.
f. Make aliquots and store at -80˚C. Upon thawing, do not refreeze and use within 4 weeks.

3. Ovotransferrin, also referred to and sold as conalbumin (Sigma-Aldrich: C7786). Prepare 5 ml of 10 mg/ml ovotransferrin solution as follows:
   a. Add 50 mg of ovotransferrin to 5 ml of KO-DMEM.
   b. Put on a roller for up to 20 minutes or until the solution is clear for complete dissolution.
   c. Filter the clear solution through a 0.22 µm syringe filter.
   d. Aliquot and store at 4˚C.
4. Chicken serum (Biosera: CH-515 or Sigma-Aldrich: C5405).

2.5 Complete PGC culture medium

Prepare any of the following complete PGC culture medium by adding the following growth factors (Whyte et al., 2015).

1. Prepare 10 ml of FAOT complete PGC medium by adding the following growth factors (**see Note 4**):
   a) 10 ml of PGC basal medium.
   b) 1.6 µl of human FGF2 (25 µg/ml).
   c) 10 µl of human Activin A (25 µg/ml).
   d) 10 µl of Ovotransferrin (10 mg/ml).
   e) 2.0 µl of 5000X Vitamin B12.
2. Prepare 10 ml of FACSOT complete PGC medium by adding the following growth factors (**see Note 5**):
   a) 10 ml of PGC basal medium.
   b) 1.6 µl of human FGF2 (25 µg/ml).
   c) 10 µl of human Activin A (25 µg/ml).
   d) 20 µl of chicken serum.
   e) 5 µl of Ovotransferrin (10 mg/ml).
   f) 2.0 µl of 5000X Vitamin B12.
3. Prepare 10 ml of FABOT complete PGC medium by adding the following growth factors (**see Note 6**):
   a) 10 ml of PGC basal medium.
   b) 1.6 µl of human FGF2 (25 µg/ml).
   c) 10 µl of human Activin A (25 µg/ml).
   d) 10 µl of human BMP4 (25 µg/ml).
   e) 10 µl of Ovotransferrin (50 ng/µl).
   f) 2.0 µl of 5000X Vitamin B12.

2.6 PGC derivation, culture and cryopreservation

1. For blood-derived PGCs, incubated chicken eggs containing embryos at Hamburger and Hamilton (HH) stages 15 – 16^{+} (Hamburger & Hamilton, 1951).
2. Scissors and tweezers sterilised in 70% ethanol.
3. Borosilicate microcapillary tubes (Harvard apparatus).
4. Aspirator apparatus (Sigma-Aldrich: A5177) consisting of plastic mouthpiece, latex tubing (15 inches) and silicone rubber nosepiece.
5. REDExtract-N-Amp™ Tissue polymerase chain reaction (PCR) Kit (Sigma-Aldrich; XNAT).
6. 48-well tissue culture plates (Thermo Scientific: 150687) and 24-well tissue culture plates (Thermo Scientific: 142475).
7. FAOT complete PGC medium (**See Note 7**).
8. STEM-CELLBANKER® (Amsbio: 11897). Alternatively, use locally prepared cryopreservation medium prepared as follows:
   a. 500 µl of DMSO (Sigma-Aldrich).
   b. 1 ml of chicken serum.
   c. 7.5 µl of 100 mM $CaCl_2$.
   d. 8.5 ml of KO-DMEM.
   e. Filter through a 0.22 µm syringe filter and store at -20°C.

9. 1.8 ml polypropylene cryogenic tubes (Thermo Scientific: 377267).
10. Mr. Frosty™ freezing container (Thermo Scientific: 51000-0001).

2.7 PGC transfection, selection and clonal expansion

1. Lipofectamine 2000 (Invitrogen: 11668019).
2. Opti-MEM I reduced serum medium (Invitrogen: 31985-062).
3. 0.1 mg/ml puromycin dihydrochloride (Sigma-Aldrich: P7255) dissolved in double-distilled water.
4. PX459 and PX458 CRISPR/Cas9 vectors (Idoko-Akoh et al., 2018; Ran et al., 2013).
5. 24-well tissue culture plates (Thermo Scientific: 142475), 48-well tissue culture plates (Thermo Scientific: 150687) and 96-well tissue culture plates (Thermo Scientific: 167008).

2.8 PGC injection into surrogate embryos

1. Incubated chicken eggs containing embryos at HH stages 15 – 16^{+}.
2. Scissors and tweezers sterilised in 70% ethanol.
3. Glass microcapillary tubes (Harvard apparatus, borosilicate, 1.5 mm).
4. Aspirator apparatus (Sigma-Aldrich: A5177) consisting of plastic mouthpiece, latex tubing (15 inches) and silicone rubber nosepiece.
5. 25 mM B/B compound in DMSO (Takara Bio).
6. 0.5 mM B/B compound in Ethanol (Takara Bio).
7. Penicillin-streptomycin (10,000 U/ml) (Life Technologies: 15140122).
8. Penicillin/streptomycin-B/B-compound mixture: Prepare by adding 30 µl of 0.5 mM B/B compound to 270 µl of Penicillin-streptomycin (10,000 U/ml).
9. Fast Green dye (Sigma-Aldrich F7252).
10. Leukosilk tape (BSN Medical).

2.9 Equipment and general reagents

1. Humidified incubator with 5% CO_2.
2. Category 2 biological safety cabinet.
3. Horizontal laminar flow hood.
4. Stereomicroscope set up in a horizontal laminar flow hood.
5. Egg incubator set at 37.8˚C and 50% humidity.
6. 70% ethanol.
7. Finely textured tissue paper (KIMTECH Science: 7558)
8. Sharp-tip stainless steel forceps/tweezers (~140 mm length with straight, fine pointed ends).
9. Blunt-end Stainless steel forceps/tweezers (~140 mm length with straight, blunt round ends with serrated jaws).
10. Stainless steel scissors (~140 mm length with straight, fine pointed ends).
11. 1.5 ml snap cap microcentrifuge tubes (Pyrogen-free, nuclease-free).
12. 1.5 ml screw cap microcentrifuge tubes (Pyrogen-free, nuclease-free).
13. Chicken egg holder.
14. Double-distilled water.
15. Autoclaved PBS.
16. Moving-coil microelectrode puller (Camden Instruments Ltd - model 753).
17. UV sterilizer cabinet.
18. Benchtop centrifuge (Sigma Laborzentrifugen GmbH: Model 1-14).
19. Benchtop microcentrifuge (Eppendorf 5452 MiniSpin centrifuge).
20. BD FACSAria III cell sorter (BD Biosciences).

3 Methods

3.1 Collection of blood from embryos (see Note 8)

1. In a sterile biological safety cabinet, add 500 µl of double-distilled water into each peripheral well of a 48-well tissue culture plate to reduce evaporation from the culture medium. Add 300 µl of FAOT medium into a well in the plate and repeat this for the number of desired wells.
2. Pull microcapillary tubes using a moving-coil microelectrode puller to create needles. Sterilise the pulled microcapillary tubes under UV in a UV sterilizer cabinet (**Figure 2**).
3. Fertile chicken eggs are incubated for 2.5 days to obtain stage 16 HH embryos. Embryos can be between stages 15-16^{+} HH but not older than stage 17 HH.
4. Sterilise the stereomicroscope and other tools in the horizontal laminar flow hood using 70% ethanol.
5. Using the stereomicroscope, break the pulled end of the microcapillary needless using sterilised sharp-tip forceps to create a narrow opening (~100 µm). Insert the unpulled end of the microcapillary tube into the aspirator tube. Insert a sterile 1.0 ml filter pipette tip into the other end of the aspirator tube. Ensure that the exposed needle end of the microcapillary tube and the pipette tip do not touch laboratory surfaces (**Figure 3**).
6. Take an egg from the incubator and sterilise by lightly wiping using 70% ethanol and gloved hands. Using the blunt-end forceps, create a window on the blunt end of the egg.
7. Using a sterile blunt-end forceps, gently remove the shell membrane to expose the embryo. The heart should be visibly beating for efficient sampling of embryonic blood.
8. Blow a small bubble in the egg albumin to demonstrate that the microcapillary needle is patent.
9. Insert the needle into the dorsal aorta at a shallow angle (10 – 30˚) and aspirate 1 - 2 µl of embryonic blood into the microcapillary tube.
10. Gently express the aspirated blood into the FAOT medium in a well of the 48-well plate. Repeat this procedure for the desired number of embryos. Do not mix blood samples from different embryos in a well if the goal is to obtain pure PGC lines.
11. Collect embryonic tissue from sampled embryos for extraction of genomic DNA to perform W chromosome PCR sexing: Stabilise the embryo with the blunt-end forceps and use the fine forceps to tear a small piece of the visible vitelline membrane and underlying tissues. Place the excised tissue in a sterile 1.5 ml snap cap microcentrifuge tube, and store at -20˚C prior to genomic DNA extraction and subsequent PCR-aided sex determination. Wash down the forceps and scissors with sterile PBS and then 70% ethanol between embryos.
12. At the end of the experiment, wash the aspirator tube in tap water and spray with 70% ethanol. Allow to airdry before storage.

3.2 PCR-aided determination of the sex of chicken embryos

The sex of a PGC culture can be determined by sexing the embryo that was sampled. Use the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich; XNAT) for sex screening of embryos as follows:

1. Add 100 µl of Extraction solution to the embryonic tissue collected in sterile 1.5 ml snap cap microcentrifuge tube.
2. Add 25 µl of Tissue Preparation solution.
3. Vortex and then incubate at room temperature for 10 minutes.
4. Pierce the lid of the closed microcentrifuge tube and heat at 95˚C for 3 minutes.
5. Add 100 µl of Neutralization solution.
6. Spin the sample for 2 minutes at 13000 revolutions per minute (rpm) (equivalent to 12,470 g) in a benchtop centrifuge and gently collect the lysate.
7. Make a 1:10 dilution of the lysate in nuclease-free water for PCR.
8. Prepare PCR reaction using REDExtract PCR mix as follows (consult the manufacturer's manual if needed):

Reagent	**Volume (µl)**
REDExtract PCR mix	10.0
Left primer (50 pmol/µl)	0.4
Right primer (50 pmol/µl)	0.4
Nuclease-free water	5.2
Sample lysate (1:10 dilution)	4.0
Total volume	**20.0**

9. PCR primers are designed to amplify a repeated section of the female W sex chromosome which is present in many copies. Amplification of a section of the W chromosome may be performed using the following primer pair:
    Left primer: 5' – CCCAAATATAACACGCTTCACT – 3'
    Right primer: 5' – GAAATGAATTATTTTCTGGCGAC – 3'
10. Use the following thermal cycling profile for the W chromosome primer pair above:
    a. 94˚C for 5 minutes.
    b. 35 cycles of 94˚C for 30 seconds, 60˚C for 30 seconds and 72˚C for 30 seconds.
    c. 60˚C for 10 minutes.
11. Include a no-template negative control, a positive male DNA control and positive female DNA control.
12. Run the PCR reaction on 1% agarose gel in 1X TAE buffer using an electrophoresis condition of 100 volts for 1 hour. A PCR product of approximately 200 base-pairs will only be present in female samples.
13. Also perform PCR amplification of a housekeeping gene to confirm the integrity of the DNA samples. Amplification of the GAPDH gene may be performed using the following primer pair:
    Left primer: 5' – TGTGACTTCAATGGTGACA – 3'
    Right primer: 5' – CAGATCAGTTTCTATCAGC – 3'
14. Include the controls used for the W PCR and use the following thermal cycling profile for the GAPDH primer pair above:
    d. 94˚C for 5 minutes.
    e. 35 cycles of 94˚C for 30 seconds, 60˚C for 30 seconds and 72˚C for 30 seconds.
    f. 60˚C for 10 minutes.
15. Perform gel electrophoresis as described above. The PCR product for the GAPDH primer pair is 700 base-pairs and should be present in all samples except the negative no-template control. If the GAPDH PCR product is absent in a tissue sample, then the DNA may be degraded. The sex PCR assays can also be performed on genomic DNA collected from PGCs, but the rate of success of culture derivations for male and female embryos sampled cannot be determined if the embryos are not sexed.

3.3 *In vitro* propagation of PGCs

1. The 1 – 2 µl embryonic blood is added to the 300 µl of FAOT culture medium in a single well of the 48-well plates. Transfer the plate to a humidified incubator set at 37˚C and 5% CO_2.
2. Refresh the culture medium every 48 hours. Without disturbing the cells aggregated in the bottom centre of the well, remove 90 µl of the culture medium by placing the pipette tip against the wall of the well. Replenish the medium by gently adding 100 µl of fresh FAOT medium.
3. PGCs are visible after a week and several hundred cells will be apparent by week two (**Figure 4**). Blood cells are mostly lysed by week three.
4. After about a week, the culture medium can be pipetted up and down 10 times to break up PGC clumps but only after refreshing the medium. Repeat if necessary during subsequent feedings.
5. Count cells in cultures at 3 – 4 weeks and determine the cell number. Cultures containing more than 50,000 cells are successful derivations (**see Note 9**).
6. After 4 weeks, discard cultures in 48-well plates that do not contain up to 50,000 PGCs. These are failed derivations.
7. The colour of the culture medium changes to yellow (becomes acidic) as PGCs reach confluency. PGCs in confluent wells (containing > 50,000 cells) should be transferred to a 24-well plate.
8. The PGC cultures are propagated in 500 – 600 µl FAOT medium/well in 24-well plates. Add 1 ml of double-distilled water into each peripheral well of a 24-well plate to reduce evaporation from the culture medium. Top up the transferred PGC culture to 500 µl with fresh FAOT medium.
9. Refresh the culture medium every two days:
    a. Gently pipette up and down five times without forming bubbles.
    b. Transfer medium to a sterile 1.5 ml screw-cap microcentrifuge tube.
    c. Centrifuge in a benchtop microcentrifuge at 1,600 rpm (200 g) for 4 minutes. This is the standard

centrifugation condition for PGCs.

d. Carefully remove the supernatant and resuspend the visible cell pellet in 500 µl of fresh FAOT medium.

10. Propagate PGCs to a maximum density of 300,000 cells/well in a 24-well plate. Either split into two or three wells at a seeding density of 50,000 cells/well. Alternatively, discard or cryopreserve excess PGCs.

3.4 Cryopreservation of PGCs

1. Only the PGC cultures that are free of contamination, healthy (> 90% cell viability) and of optimal confluency should be cryopreserved. For optimum results, cells should be in log phase of growth with 50 – 80% confluency representing approximately 120,000 to 200,000 cells in 500 µl of FAOT medium in a well of a 24-well plate.
2. Count the number of cells in the culture. Gently pellet the cells by standard centrifugation, remove supernatant, and gently resuspend pellet in STEM-CELLBANKER® cryopreservation medium at a concentration of 100,000 cells/100 µl. If using locally prepared 5%DMSO-10%FBS cryopreservation medium, add an equal volume of cryopreservation medium at room temperature dropwise to the cells (100,000 cells/100 µl) to prevent cell lysis.
3. Dispense at least 200 µl of PGC cryopreservation mixture into a 1.8 ml polypropylene cryogenic tube.
4. Place the cryogenic tubes in a Mr. Frosty™ freezing container for controlled cooling (at the rate of -1˚C/minute) to -80˚C.
5. After 6 hours of storage at -80˚C, transfer the frozen cryogenic tubes to an ultra-low temperature freezer for long-term storage at < -150˚C. Avoid storing PGCs at -80˚C for more than 1 week as this may reduce viability upon thawing.

3.5 Thawing of PGCs

1. Thaw the cryogenic tube containing the frozen PGCs at room temperature or by holding in your gloved hand.
2. Add room-temperature PGC basal medium (4 times the original volume of the cryopreservation mixture) to the frozen cells in a dropwise manner. For instance, if the volume of cryopreservation mixture in the cryogenic tube is 200 µl, add 800 µl of PGC basal medium.
3. Allow to stand at room temperature for up to 5 minutes and gently mix the cell suspension.
4. Transfer the thawed cell suspension into a 1.5 ml microcentrifuge tube and pellet the cells by standard centrifugation.
5. Remove the supernatant and gently resuspend the cell pellet in 500 µl of FAOT medium. Transfer the cell suspension to a well in a 24-well plate. Add 1 ml of double-distilled water to each peripheral well of the 24-well plate to reduce evaporation from the culture medium and place in a humidified incubator set at 37˚C and 5% CO_2 to culture PGCs as described previously.

3.6 Transfection of PGCs

1. 100,000 cells (but do not exceed 150,000 cells) are used per transfection (**see Note 10**).

2. Add 2 µg (up to 4 µg can be used) of plasmid DNA to be transfected to approximately 150 µl Opti-MEM I medium. If transfecting short single stranded DNA oligonucleotides (ssODN), do not use more than a total of 400 ng or 10 µM per transfection to prevent toxicity (**see Note 11** for use of ssODN templates). The total volume of the DNA/Opti-MEM mixture will be 150 µl in a 1.5 ml screw-cap microcentrifuge tube.

3. In separate 1.5 ml screw-cap microcentrifuge tube, add 2 µl (3 µl is also suitable) of Lipofectamine 2000 to 148 µl (or 147 µl if using 3 µl of Lipofectamine 2000) of Opti-MEM I medium to have a total volume of 150 µl. Let the Lipofectamine/Opti-MEM mixture incubate at room temperature for 15 - 20 minutes.

4. Gently combine the DNA/Opti-MEM mixture with the Lipofectamine/Opti-MEM mixture to have a total volume of 300 µl. Gently pipette the DNA/Lipofectamine mixture up and down five times and incubate for 25 minutes at room temperature. Do not vortex or mix violently.

5. While the Opti-MEM mixture is incubating, pellet the cells to be transfected by standard centrifugation at 1,600 rpm (200 g) for 4 minutes. Remove the supernatant.

6. Gently resuspend the cell pellet in 750 µl of Opti-MEM I medium and centrifuge at 1,600 rpm (200 g) for 4 minutes to wash the cells. Remove the supernatant.

7. Gently resuspend cell pellet at a concentration of 100,000 cells per 50 µl of Opti-MEM I medium (do not exceed 150,000 cells).

8. Add 50 µl of the Opti-MEM I cell suspension to the 300 µl DNA/Lipofectamine Opti-MEM mixture and gently pipette up and down five times. The transfection mixture has a final volume of 350 µl in a 1.5 ml screw-cap microcentrifuge tube. Do not vortex or mix violently.

9. Incubate the transfection mixture with the microcentrifuge tube standing upright in an incubator at 37˚C and 5% CO_2 for 6 hours to overnight. The screw cap should be set on loosely to allow CO_2 and oxygen to enter the microcentrifuge tube through the threads.

10. Tighten the screw cap on the microcentrifuge tube and centrifuge the transfection mixture at 2,200 rpm (300 g) for 10 minutes to pellet the cells. A cell pellet may not be visible.

11. Remove as much of the supernatant from the microcentrifuge tube as the transfection solution is toxic.

12. Gently resuspend the cell pellet in 500 µl of room-temperature FAOT medium and transfer to a single well in a 24-well plate. Add 1 ml of double-distilled water to each peripheral well of the 24-well plate to reduce evaporation from the culture medium and place in a humidified incubator set at 37˚C and 5% CO_2.

3.6.1 Selection for CRISPR-transfected cells (see Note 12)

1. For CRISPR/Cas9 plasmids expressing a fluorescent reporter protein, a high level of expression should be achieved between 48 and 72 hours after transfection. Fluorescence-activated cell sorting may be performed within this period to select for transfected cells. Single cell sorting may be directly performed at this point but the efficiency and cell survival may be low. We recommend sorting and expanding the mixed pool of transfected cells to allow recovery and optimal cell health before performing single cell cultures.

2. If the CRISPR/Cas9 plasmid bears an antibiotic selection gene, the selection reagent may be added 24 hrs after transfection (**see Note 13**).

3. Before adding the selection reagent, the culture medium may be refreshed if the cells have not been perturbed by slowly and gently withdrawing 250 µl of the culture medium by directing the pipette tip at the wall of the well. Centrifuge this solution at 1,600 rpm (200 g) for 4 minutes and discard the supernatant. Resuspend any pellet with 260 µl of FAOT medium and add this solution back to the side of the well.

4. For CRISPR/Cas9 plasmids bearing a puromycin-resistance gene, add 2.0 µl of 0.1 mg/ml puromycin solution to the transfected cells and incubate for 48 hours (**see Note 14**).

5. Centrifuge the transfected cells at 1,600 rpm (200 g) for 4 minutes. Discard the supernatant and resuspend the cell pellet in 500 µl of PGC basal medium.

6. Centrifuge the cells again for 1,600 rpm (200 g) for 4 minutes. Discard the supernatant, resuspend the cell pellet in 500 µl of fresh FAOT medium and transfer to a new well in a 24-well plate. If there is massive cell death and very few live cells, resuspend the puromycin-treated cells in 300 µl of fresh FAOT medium and culture in a 48-well plate. Add 500 µl of double-distilled water into each peripheral well of the 48-well tissue culture plate to reduce evaporation from the culture medium. Place the plate in a humidified incubator set at 37˚C and 5% CO_2 and continue to culture.

7. Refresh the culture medium every 48 hours:
    a. 24-well plate: without disturbing the cells aggregated in the centre of the well, remove 250 µl of the culture medium by placing the pipette tip at wall of the well. Centrifuge at 1,600 rpm (200 g) for 4 minutes. Discard the supernatant and resuspend any pellet in 260 µl of FAOT medium. Gently adding this solution back to the side of the well.
    b. 48-well plate: without disturbing the cells aggregated in the centre of the well, remove 90 µl of the culture medium by placing the pipette tip at wall of the well. Replenish the medium by gently adding 100 µl of fresh FAOT medium. Transfer the culture to a 24-well plate when the cell count is > 50,000 cells.
8. Untransfected PGCs that are not expressing the puromycin-resistance gene will die out within 5 days from the time of the addition of puromycin. The culture medium will contain a lot of visible cellular debris and dead cells. Clumps of living cells will gradually become apparent and may be clonal populations.

9. Propagate PGCs to a maximum density of 300,000 cells/well in a 24-well plate. Split into two or three wells at a seeding density of 50,000 cells/well to continue expansion. Collect cell pellets for genomic DNA extraction to analyse gene editing. Cryopreserve the remaining PGCs as described previously.

3.7 Genome analysis of transfected PGCs

1. Use >100,000 cells for extraction of genomic DNA.
2. Propagate PGCs to a maximum density of 300,000 cells/well in a 24-well plate.
3. Centrifuge the PGCs at 1,600 rpm (200 g) for 4 minutes. Discard the supernatant. The cell pellet can be stored at -20˚C until ready for use.
4. Use the QIAMP DNA Micro kit (Qiagen; 56304) to extract genomic DNA according to the manufacturer's instruction. PCR amplification of the target site is performed using the purified DNA.
5. Gene deletions using two CRISPR/Cas9 gRNAs may be immediately assessed by running the PCR products in 1 – 2% agarose gels and checking for the estimated difference in product size comparing with PCR products from wildtype cells.
6. Single-site targeting may be assessed by performing Sanger sequencing of the PCR products. The online TIDE analysis suite (https://tide.nki.nl/) can be used to estimate INDEL frequency through analysis of the Sanger sequencing chromatogram files (.AB1 file format).
7. Proceed to single-cell clonal culture.

3.8 Single-cell clonal culture

1. Use expanded transfected PGC cultures that are free of contamination, healthy (> 90% cell viability) and of optimal confluency. For optimum results, cells should be in the log phase of growth with 50 – 80% confluency representing approximately 120,000 to 200,000 cells in 500 µl of FAOT medium in a well of a 24-well plate (**see Note 15**).
2. Add 200 µl of double-distilled water into each peripheral well of a 96-well tissue culture plate to reduce evaporation from the culture medium. Add 50 µl of FAOT medium into each of the inner 60 wells in the plate. Conditioned FAOT medium or 50% conditioned FAOT medium (prepared by mixing conditioned FAOT medium with fresh FAOT medium) may be used throughout for single cell culture maintenance when cell growth appears to be retarded (**see Note 16**).
3. Single cell plating may be performed manually by hand through serial dilution until a single cell is seeded in a well of a 96-well plate. Allow the plate to sit for 10 minutes and then observe under the microscope to ensure that each selected well contains a single PGC (**see Note 17**).
4. Alternatively, single cell plating may be easily and quickly performed using the BD FACSAria III cell sorter. Seed a single PGC into each well containing 50 µl of FAOT medium in a 96-well plate. Transfer the plate into a humidified incubator set at 37˚C and 5% CO_2 and incubate for 48 hours.
5. Add 50 µl of FAOT medium to each well for a total culture volume of 100 µl and incubate for another 48 hours.
6. Again, add 50 µl of FAOT medium to each well to achieve a total culture volume of 150 µl and incubate for another 48 hours.
7. Subsequently, the culture medium may be refreshed every 48 hours by slowly and gently withdrawing 45 µl of the culture medium by directing the pipette tip at the wall of the well. Gently replace with 50 µl of fresh FAOT medium. Successful cultures in 96-well plates take 2 to 3 weeks to reach approximately 30 – 50% confluency.
8. Once the cell confluency reaches approximately 50%, transfer the PGC culture to a well in a 48 well-plate and increase the culture volume to 300 µl with fresh FAOT medium. Add 500 µl of double-distilled water into each peripheral well of the 48-well tissue culture plate to reduce evaporation from the culture medium. Transfer the plate into a humidified incubator set at 37˚C and 5% CO_2.
9. Refresh the culture medium every 48 hours. Without disturbing the cells aggregated in the centre of the well, remove 90 µl of the culture medium by placing the pipette tip at wall of the well. Replenish the medium by gently adding 100 µl of fresh FAOT medium.
10. After about a week, the culture medium can be pipetted up and down five times to break up PGC clumps but only after refreshing the medium.
11. Count the cells in the cultures. Cultures with more than 50,000 cells are successful clonal derivations. The colour of the culture medium changes to yellow (becomes acidic) as PGCs reach confluency. Successful clonal derivations take 1 week to reach 50,000 cells in a 48-well plate upon transfer from a 96-well plate.
12. Transfer PGCs in confluent wells (containing > 50,000 cells) of the 48-well plate to a 24-well plate. Increase the volume of each PGC culture to 500 µl/well with fresh FAOT medium. Add 1 ml of double-distilled water into each peripheral well of the 24-well plate to reduce evaporation from the culture medium.

13. Propagate the clonal PGCs to a maximum density of 300,000 cells/well in the 24-well plate. Split into two or three wells at a seeding density of 50,000 cells/well. Collect cell pellets for genomic DNA extraction to confirm gene editing. Cryopreserve the remaining PGCs as described above.
14. Overall, it takes 3 – 5 weeks to establish a clonal line from a single PGC. Higher efficiency is obtained for cloning male PGCs compared to female PGCs.

3.9 Injection of PGCs into surrogate host embryos

1. Thaw the cryopreserved clonal PGCs 5 to 7 days before the intended injection date and propagate to a maximum density of 300,000 cells/well in a 24-well tissue culture plate.
2. Pull microcapillary tubes using a moving-coil microelectrode puller to create pointed needles. Sterilise the pulled microcapillary tubes under UV in a UV sterilizer cabinet.
3. Fertile chicken eggs are incubated upside down (pointy end up) for 2.5 days to obtain stage 16 HH embryos. Embryos can be between stages 15-16+ HH but not older than stage 17 HH.
4. Surface-sterilise the stereo microscope and other tools in the horizontal laminar flow hood using 70% ethanol.
5. Under the stereo microscope, break off a small portion of the pulled end of the microcapillary tube using sterilised sharp-tip forceps to create a needle. Insert the unpulled end of the microcapillary tube into the aspirator tube. Insert a sterile 1 ml filter pipette tip into the other end of the aspirator tube. Ensure that the exposed needle end of the microcapillary tube and the pipette tip do not touch the laboratory surfaces. Alternatively, a needle beveller can be used to create a bevelled opening.
6. The cultured PGCs are pelleted by standard centrifugation and re-suspended in KO-DMEM at a concentration of 5,000 cells/µl.
7. (Optional) If using iCaspase9 sterile embryos, add 1.0 µl of B/B compound to 50 µl of the PGC suspension and maintain at room temperature.
8. A neutral dye solution such as Fast Green dye (Sigma) is added to the PGC suspension to aid visualisation of the injection. Add 0.5 µl of 0.1% dye to 50 µl of PGC suspension.
9. Take an egg from the incubator and sterilise lightly using 70% ethanol egg. Using the blunt-end forceps, create a small window on the pointy end of the egg.
10. Using a sterile blunt-end forceps, gently remove the shell membrane to expose the embryo. The heart should be visibly beating.
11. (Optional) Prior to injection, aspirate 1 µl of endogenous blood to make space for the injection in the circulatory system of the embryo.
12. Flick PGC suspension to resuspend PGCs as the cells settle rapidly.
13. Aspirate 1 – 2 µl of the PGC suspension into the microcapillary tube. If PGC solution will not enter the needle, break off a small portion of the tip and repeat aspiration.
14. Insert the needle into the dorsal aorta at a shallow angle (10 – 30°) and inject the PGC suspension into the vascular system. The dye should enable visualisation of the vascular system filling with the PGC solution.
15. If using iCaspase9 sterile hosts, gently inject 50 µl of penicillin/streptomycin-B/B-compound mixture on top of the embryo. For other embryos, use 50 µl penicillin/streptomycin solution.
16. Seal the egg with Leukosilk tape and incubate the manipulated egg blunt end up with rocking until hatching.
17. Carefully remove the microcapillary tube and dispose in a sharps bin.
18. After successful hatching, collect chorio-allantoic membrane (CAM) samples from each egg for DNA extraction for sex determination of the surrogate host as described in **section 3.2**.
19. Raise hatched chicks to sexual maturity and breed to generate the G1 generation of genome-edited chickens.

Notes

Note 1: We advise that 1 ml aliquots of 100X EmbryoMax® nucleosides (Merck Millipore: ES-008-D) should be made and stored at -80˚C. Avoid re-freezing.

Note 2: Vitamin B12 can be added directly to the PGC basal medium or alternatively to the complete PGC culture. Do not add to both as the final concentration in the complete PGC culture must be 0.0068 µg/ml. For example, 10 µl of

5000X vitamin B12 solution can be added to 50 ml of PGC basal medium or alternatively, it can be added to 50 ml of complete PGC culture.

Note 3: The final concentration of BSA in 1 ml of 25-µg/ml FGF2 solution will be 0.125%, with 100 mM NaCl.

Note 4: 'FAOT' is an abbreviation derived from the growth factors used in the preparation of the medium; FGF2 (F), Activin A (A) and Ovotransferrin (OT)

Note 5: 'FACSOT' is an abbreviation derived from the growth factors used in the preparation of the medium; FGF2 (F), Activin A (A), Chicken Serum (CS) and Ovotransferrin (OT)

Note 6: 'FABOT' is an abbreviation derived from the growth factors used in the preparation of the medium; FGF2 (F), Activin A (A), BMP4 (B) and Ovotransferrin (OT)

Note 7: We routinely use FAOT serum-free medium. Use of FACSOT or FABOT medium gives the same culture efficiency in our hands but may be more suitable for some PGC lines.

Note 8:

a. Chicken eggs and embryos usually do not usually contain infectious material and so are not a biohazard. However, appropriate care must be taken and appropriate personal protection must be worn during the procedure (eye protection, gloves, protective coat).

b. The reusable parts of the aspirator apparatus must be rinsed (tap H20), sprayed with 70% ethanol, and air dried on a finely textured tissue paper before use. The same decontamination protocol must be used when finished to eliminate the possibility of bacterial and egg product contamination.

c. Glass microcapillaries must be safely disposed in a sharps bin immediately after use. Care must be taken not to leave used microcapillaries on bench tops.

d. Care must be taken not to let the mouthpiece and needle come in contact with any laboratory surfaces. Even contact with finely textured tissue paper could pose a problem. It is good practice to drape the aspirating apparatus over the microscope eyepieces so that neither end touches a laboratory surface.

e. Care must be taken not to touch egg contents with gloves and then onto mouthpiece. Raw egg products may contain salmonella and should be treated with care.

Note 9: If the PGC derivation is fast growing, cells may need to be counted at two weeks and transferred to a 24-well plate or they will become too confluent and die by three weeks of culture.

Note 10: Using less than 100,000 cells will not pellet well when cells are centrifuged after the 6-hour Opti-MEM I incubation step and will also give a lower transfection efficiency. PGCs to be transfected should be in the log phase of growth and around 50 – 80% confluency representing approximately 120,000 to 200,000 cells in 500 µl of FAOT medium in a well of a 24-well plate.

Note 11: Use of CRISPR/Cas9 and ssODN repair templates for performing small sequence changes (Idoko-Akoh et al., 2018);

a. Design a repair template varying in length from 70 – 140 base-pairs.
b. Synthesize with Integrated DNA technologies (IDT) and purchase as desalted 4nM ultramer™ oligonucleotides. Use other vendors if preferred.
c. Briefly centrifuge the tube before opening to avoid losing dried pellets and resuspend to a stock concentration of 100 µM by adding 40 µl of TE buffer in a sterile microsafety cabinet. Briefly centrifuge the resuspended ssODN at high speed. Ultramer™ oligonucleotides may also be purchased as 100 µM suspensions in TE buffer.
d. If using a single ssODN repair template, dilute a small amount of the stock solution to 10 µM using TE buffer and use 1 µl per transfection. If performing two different targeting, make 5 µM aliquots of each ssODN by diluting with TE buffer and use 1 µl of each 5 µM ssODN per transfection. In our hands, 1 µM

also produced a good gene editing efficiency comparable with using 10 µM. This makes it theoretically possible to design multiple ssODN repair templates to target multiple genomic locations. Do not exceed 10 µM per transfection because ssODN are toxic to cells in large amounts.

e. Proceed by mixing 1.0 to 1.5 µg of PX459 or PX458 CRISPR/Cas9 plasmid with 10 µM of ssODN if using only one repair template. If using a mixture of two repair templates, use 5 µM of each at 1:1 ratio as illustrated below:

    i. 10 µM ssODN + 1.5 µg PX459 plasmid or

    ii. 5 µM ssODN1 + 5 µM ssODN2 + 1.5 µg PX459 plasmid

Note 12: We use the PX458 and PX459 wildtype CRISPR/Cas9 vectors from Feng Zhang's lab (Ran et al., 2013). PX458 vector expresses the eGFP protein while PX459 (V2.0) expresses the puromycin-resistance protein. To increase the efficiency of ssODN-mediated homology-directed repair, we use the HF-PX459 (V2.0) vector which expresses the high-fidelity Cas9-HF1 protein and puromycin-resistance gene (Idoko-Akoh et al., 2018).

Note 13: The 24-hour timepoint after transfection is determined from the time the transfected cells are resuspended in FAOT culture medium and placed into the incubator set at 37˚C and 5% CO_2.

Note 14: 2.0 µl of 0.1 mg/ml puromycin is quantitated to kill 99% of many PGC lines in 500 µl culture containing 150,000 cells after 48 hours of incubation. Some PGC lines may be more or less sensitive to puromycin and may require optimisation.

Note 15: Do not isolate single cell cultures from overgrown and highly confluent cultures as cell growth would be within the stationary and decline phases. There is a significant reduction in the number of viable cells in these phases which significantly reduces the success rate in establishing viable single cell cultures.

Note 16: FACSOT, FABOT or conditioned FAOT medium may be used for PGC lines that are difficult to grow as single cells. Prepare conditioned FAOT medium as required and use immediately. To prepare conditioned medium:

a. Add 500,000 PGCs to 1 ml of FAOT culture medium in a well of a 12-well tissue culture plate.

b. Add 1.5 ml of double-distilled water to the peripheral wells of the plate and incubate for 24 hours at 37°C and 5% CO_2 in a humidified incubator.

c. Centrifuge the culture at 1700 rpm for 4 minutes to collect the culture supernatant.

d. Filter the supernatant through a 0.22 µm syringe filter (Merck Millipore; SLGPO33RS).

e. Store the filtered supernatant at 4°C and use within 3 days.

Note 17: Performing single cell plating manually can be tedious, time-consuming and inefficient. We strongly recommend the use of a cell sorting machine such as the BD FACSAria III cell sorter.

Figure 1. Workflow for the generation of genome-edited (GE) chickens through targeting of cultured PGCs.

Figure 2. Pulled microcapillary tube. Green arrow points to the wide end of the microcapillary tube that is inserted into the aspirator tube. To aspirate 1 – 2 µl of blood or PGC suspension, do not exceed the point indicated by the red arrow on the needle end.

Figure 3. Aspirator apparatus draped over the eyepiece of a stereo microscope **a,** positioned in a sterile horizontal laminar flow hood. The aspirator tube may be fitted with a 0.2 µm filter unit **b**, Insert the wide end of the pulled microcapillary tube into the aspirator tube on one end **c**,. Use a sterile pair of forceps to break part of the needle end of the capillary to make it patent. Dissecting lights **d**, provide illumination for good visibility. Place the egg in an egg-stand **e**, to stabilise it. Drape the assembled aspirator apparatus over the microscope to prevent it from coming in contact with laboratory surfaces. Insert a 1 ml filter pipette tip **f**, into the other end of the aspirator tube which will be used to aspirate blood or inject PGCs. Use this positioning during blood collection and PGC injection experiments.

Figure 4. Micrograph of PGCs in culture. a, 4x magnification. **b,** 10x magnification. **c,** 40x magnification: black arrowhead shows a PGC with smooth cell membrane. Blue arrowhead shows a PGC with blebs bulging from the cell membrane. Scale bar – 50 µm.

Acknowledgment

Illustrations in Figure 1 depicting PGC derivation from embryos, CRISPR/Cas9 gene editing, PGC injection into embryos and GE chicks were created with BioRender.com and are used under license.

References

Ballantyne, M., Woodcock, M., Doddamani, D., Hu, T., Taylor, L., Hawken, R. J., & McGrew, M. J. (2021). Direct allele introgression into pure chicken breeds using Sire Dam Surrogate (SDS) mating. *Nature Communications*, *12*(1), 1–10. https://doi.org/10.1038/s41467-020-20812-x

Bravo, G. A., Schmitt, C. J., & Edwards, S. V. (2021). What Have We Learned from the First 500 Avian Genomes? *Https://Doi.Org/10.1146/Annurev-Ecolsys-012121-085928*, *52*, 611–639. https://doi.org/10.1146/ANNUREV-ECOLSYS-012121-085928

Hamburger, V., & Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *Journal of Morphology*, *88*(1), 49–92. https://doi.org/10.1002/JMOR.1050880104

Idoko-Akoh, A., Taylor, L., Sang, H. M., & McGrew, M. J. (2018). High fidelity CRISPR/Cas9 increases precise monoallelic and biallelic editing events in primordial germ cells. *Scientific Reports*, *8*(1), 15126. https://doi.org/10.1038/s41598-018-33244-x

Ioannidis, J., Taylor, G., Zhao, D., Liu, L., Idoko-Akoh, A., Gong, D., Lovell-Badge, R., Guioli, S., McGrew, M. J., & Clinton, M. (2021). Primary sex determination in birds depends on DMRT1 dosage, but gonadal sex does not determine adult secondary sex characteristics. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(10). https://doi.org/10.1073/PNAS.2020909118/-/DCSUPPLEMENTAL

Lee, J. H., PARK, J. J.-W. J. J.-W., KIM, S. W., PARK, J. J.-W. J. J.-W., & Park, T. S. (2017). C-X-C chemokine receptor type 4 (CXCR4) is a key receptor for chicken primordial germ cell migration. *Journal of Reproduction and Development*, *63*(6), 555–562. https://doi.org/10.1262/jrd.2017-067

Oishi, I., Yoshii, K., Miyahara, D., Kagami, H., & Tagami, T. (2016). Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Scientific Reports*, *6*, 23980. https://doi.org/10.1038/srep23980

Park, T. S., Lee, H. J., Kim, K. H., Kim, J.-S., & Han, J. Y. (2014). Targeted gene knockout in chickens mediated by TALENs. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(35), 1–6. https://doi.org/10.1073/pnas.1410555111

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, *8*(11), 2281–2308. https://doi.org/10.1038/nprot.2013.143

Taylor, L., Carlson, D. F., Nandi, S., Sherman, A., Fahrenkrug, S. C., & McGrew, M. J. (2017). Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development*, *144*(5), 928 LP – 934. http://dev.biologists.org/content/144/5/928.abstract

van de Lavoir, M.-C., Diamond, J. H., Leighton, P. A., Mather-Love, C., Heyer, B. S., Bradshaw, R., Kerchner, A., Hooi, L. T., Gessaro, T. M., Swanberg, S. E., Delany, M. E., & Etches, R. J. (2006). Germline transmission of genetically modified primordial germ cells. *Nature*, *441*(7094), 766–769. https://doi.org/10.1038/nature04831

Whyte, J., Glover, J. D., Woodcock, M., Brzeszczynska, J., Taylor, L., Sherman, A., Kaiser, P., & McGrew, M. J. (2015). FGF, Insulin, and SMAD Signaling Cooperate for Avian Primordial Germ Cell Self-Renewal. *Stem Cell Reports*, *5*(6), 1171–1182. https://doi.org/10.1016/j.stemcr.2015.10.008

Zhang, G. (2015). Bird sequencing project takes off. *Nature 2015 522:7554*, *522*(7554), 34–34. https://doi.org/10.1038/522034d